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TITLE: Loss of PTEN as a Predictive Biomarker of REsponse to Lithium Chloride, a Potential Targeted Treatment for Breast Cancer

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14. ABSTRACT

During the first year of this project, Tasks 1a and 1b have been completed. This includes the creation of isogenic breast cell lines with heterozygous and homozygous PTEN loss, the creation of targeting vectors and the infection and screening of breast epithelial and cancer cell lines. In addition, biochemical characterization of these newly created cell lines (as well as a library of other cell lines which each represent a distinct mutation of the PI3K/mTOR pathway) has been completed.

15. SUBJECT TERMS
Isogenic cell lines, PTEN

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Introduction

The phosphatidylinositol 3-kinase (PI3K) pathway mediates key cellular functions, including growth, proliferation, survival and angiogenesis. The gene encoding the catalytic domain of PI3K, *PIK3CA*, has been found to be mutated in breast cancers at high frequency. The tumor suppressor PTEN reverses the effects of PI3K by dephosphorylating the same site on membrane phosphatidylinositols that is phosphorylated by PI3K. Genomic analysis of the PTEN gene has identified it as one of the most commonly mutated or deleted tumor suppressors in human malignancies. In breast cancer, genetic alterations of both PTEN alleles are found with a frequency of about 5%, however monoalleleic loss of PTEN is observed in as many as 50% of cases, and this can lead to aberrant PTEN signaling, resulting in early metastasis and poor prognosis.

Physiologic models of PTEN loss are needed to test potential anti-cancer therapies in preclinical animal models of breast cancer. Our lab's approach is to exploit somatic cell gene targeting to create paired isogenic cell lines with critical genetic alterations as their only differentiating factor. The changes in downstream signaling pathways can then be reliably detected, and response to new therapeutic agents can be identified. Thus, the ability to create isogenic paired human cell lines enables the evaluation of genetic alterations for predictive biomarkers of response to novel therapies.

Using gene targeting, our laboratory has introduced two common "hotspot" *PIK3CA* mutations into the MCF-10A non-tumorigenic human breast epithelial cell line. (1) Surprisingly, this study led to the discovery that GSK3beta inhibitors including lithium, an FDA approved therapy for bipolar disorders, have selective anti-neoplastic properties against human breast cancer cell lines containing oncogenic *PIK3CA* mutations. These results are now being rapidly translated into a clinical trial to determine if women with breast cancers harboring mutant *PIK3CA* will respond to lithium therapy. Because of the known opposing interactions between PI3K and PTEN, we hope to build upon this work and study the sensitivity to lithium in breast cancer cells with PTEN loss using preclinical models. If successful, we will rapidly translate these findings to an early phase clinical trial studying the safety and efficacy of oral lithium treatment in patients with breast carcinoma and examining their breast cancers for PTEN loss. We hope to provide the rationale for the use of lithium as a targeted anti-cancer treatment for breast cancer patients whose tumors harbor mutations/loss of PTEN.

Body

Statement of work

Task 1. Creation of isogenic breast cell lines with heterozygous and homozygous PTEN loss. (months 1-18)
1a. Create targeting vectors (months 1-3)
1b. Infect and screen breast epithelial and cancer cell lines (months 3-12)
1c. Biochemical and phenotypical characterization (months 12-18)
Task 2. PTEN loss sensitizes cells to lithium in vitro. (months 18-30)
2a .Expose various isogenic pairs of PTEN knock out cell lines to lithium chloride (months 18-24)
2b. Biochemical and phenotypical characterization of response to lithium chloride (months 24-30)
Task 3. PTEN loss sensitizes cells to lithium in vivo. (months 24-36)
3a. Establish xenografts of isogenic pairs of breast cancer cell lines in nude mice (months 24-30)
3b. Treatment studies using oral and intraperitoneal administration of lithium (months 28-36)

Task 1a. Create targeting vectors (months 1-3)

The grant was awarded on June 1st 2010. Dr. Higgins graduated from her Oncology Fellowship Program in Johns Hopkins Hospital on June 30th and accepted a position as a faculty member with the Breast Cancer Program at Massachusetts General Hospital on July 1 2010. A request to transfer the grant to support Dr. Higgins as she continued this work was submitted and processed in June 2010 in anticipation of this move. Johns Hopkins University has since relinquished all interest in this grant and it was transferred to Massachusetts General Hospital in September 2010. Dr. Higgins provided a detailed progress report between June 1st 2010 and Sept 30th 2010. This report covers the period from Sept 30th 2010 to June 30th 2011.

Dr. Higgins prepared and designed the planned experiments to employ gene targeting in the two breast cancer cell lines that are wild type for both *PIK3CA* and *PTEN*: HCC712 and HCC1187, which are estrogen receptor positive and negative, respectively. Targeting vectors have already been created by a former Park lab mentee (2) and were used for knocking out the *PTEN* gene in these cells.

Task 1b. Infect and screen breast epithelial and cancer cell lines (months 3-12)

Unfortunately despite several months of screening infected cells, we were unable to produce HCC712 and HCC1187 cell lines with knocked out *PTEN*. We hypothesize that this is due to the high level of genetic instability within these malignant cell lines. However; Dr. Higgins designed an alternative strategy to comprehensively study the distinct effects of several key alterations of the PI3K pathway using a library of cell lines previously created by members of the Park laboratory harboring either a *PIK3CA* mutation, (1) an *AKT1* mutation, (3) or loss of PTEN (2). Additionally Dr. Higgins and her team stably transduced Epidermal Growth Factor Receptor (EGFR) in MCF10A human breast epithelial cells using the retroviral expression vector pFBneo, which was a kind gift from Dr. Anil K. Rustgi (University of Pennsylvania). Retrovirus containing the coding sequence for EGFR was generated using Fugene6 (Roche Diagnostics, Indianapolis, IN) per the manufacturer's protocol in HEK-293T cells. Purified retrovirus was then used to infect MCF-10A cells following the manufacturer's protocol. Stable transformants were selected using 180 µg/mL G418 (Invitrogen, Carlsbad, CA). EGFR expression was confirmed by western blot using antibodies against total EGFR protein. (Figure 1) Parental MCF-10A cells were also stably transduced in parallel with an empty retroviral expression vector pFBneo (named Empty Vector or EV) and selected in the same manner to serve as controls for all experiments.

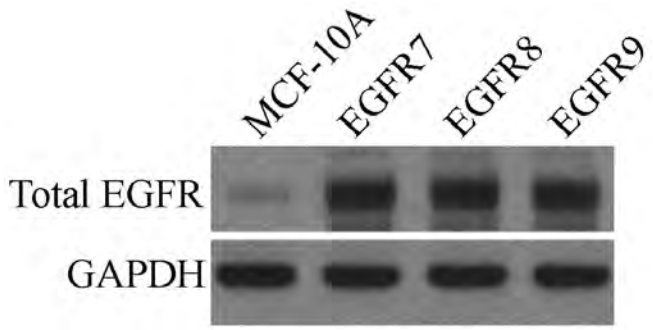

Figure 1: Overexpression of Epidermal Growth Factor Receptor in MCF-10A human breast epithelial cells. Western blot demonstrating levels of total EGFR in parental MCF-10A, and three stably transduced EGFR overexpressing clones, EGFR7, EGFR8, EGFR9. GAPDH is shown as a loading control.

We plan on using alternative strategies to pursue gene targeting of PTEN in the breast cancer cell lines and can also resort to the use of RNA interference if necessary.

Task 1c. Biochemical and phenotypical characterization (months 12-18)

Using the library of cell lines detailed above, extensive immunoblotting experiments were performed to biochemically characterize parent MCF10A cells in comparison with cell lines harboring a *PIK3CA* mutation, an *AKT1* mutation, loss of PTEN or overexpression of EGFR. We performed western blot analyses to determine the degree of MAPK and PI3K pathway activation by comparing relative levels of phosphorylated and total Akt and Erk in the absence of exogenous EGF and in the presence of physiologic concentrations of EGF.

Lysates for cells grown in each experimental condition have been prepared as previously described. (4) Western blotting was performed using the NuPage XCell SureLock electrophoresis system (Invitrogen, Carlsbad, CA) and PVDF membranes (Invitrogen, Carlsbad, CA). Primary antibodies were added overnight at 4 °C, while secondary antibodies, conjugated with horseradish peroxidase were added for 1 hr at RT. Antibodies used in this study were anti-EGFR rabbit antibody (2232; Cell Signaling Technology),

anti-phospho EGFR (Tyr 1173) rabbit anti-body (4407L; Cell Signaling Technology), anti-AKT rabbit antibody (9272; Cell Signaling Technology), anti-phospho AKT (Ser 473) rabbit antibody (9271; Cell Signaling Technology), anti-p42/p44 MAP kinase rabbit antibody (9102; Cell Signaling Technology), anti-phospho p42/p44 MAP kinase (Thr-202/Tyr-204) mouse antibody (9106; Cell Signaling Technology), anti-cyclin D1 rabbit antibody (2922; Cell Signaling Technology), anti-GSK3β rabbit antibody (9315; Cell Signaling Technology), anti-phospho GSK3β rabbit antibody (9336S; Cell Signaling Technology), and anti-GAPDH mouse antibody (6C5) (ab8245; Abcam). Blots were exposed to Kodak XAR film using chemiluminescence for detection (Perkin Elmer). All experiments were performed at least 3 times. Results of biochemical characterization of the various cell lines are shown in Figure 2.

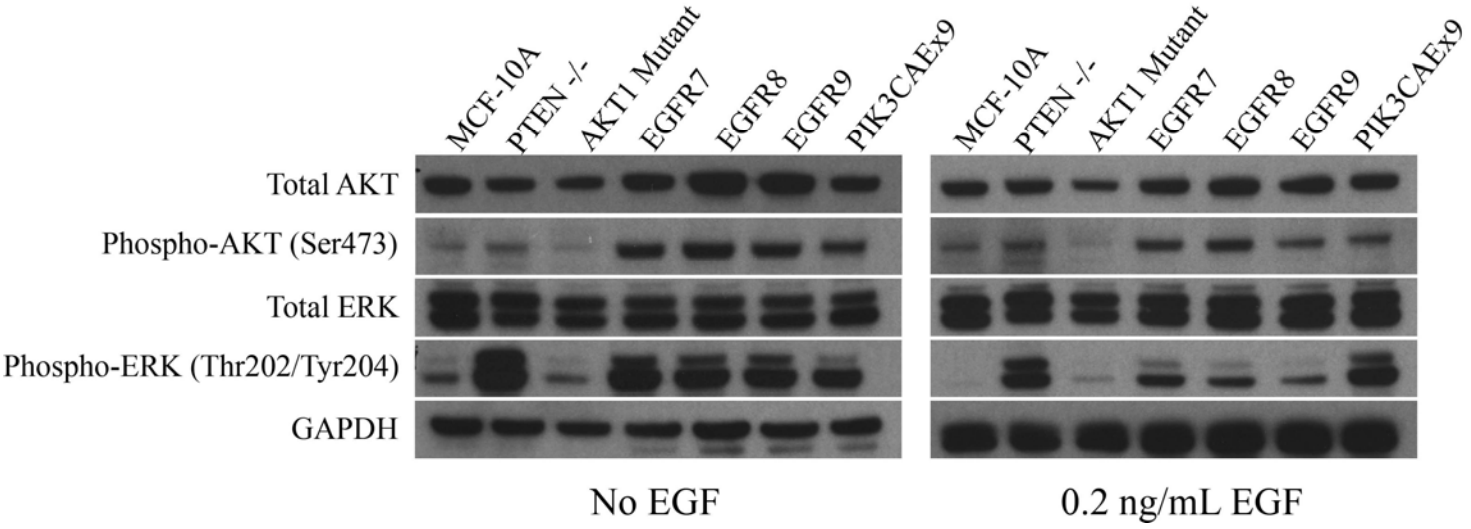

Figure 2:
Alterations in the PI3K pathway activate multiple oncogenic pathways to varying degrees. Western blot demonstrating levels of total AKT, phosphorylated AKT(Ser473), total ERK and phosphorylated ERK (Thr202/Tyr204) in parental MCF-10A, PTEN -/-, AKT1 mutant (E17K), EGFR7, EGFR8, EGFR9 (EGFR overexpressing clones) and PIK3CAEx9 (E545K) cell lines in the absence of EGF (left panel) or presence of 0.2 ng/ml EGF (right panel). GAPDH is shown as a loading control. Results are representative of multiple independent experiments.

A further part of task 1C is to perform cell proliferation assays. These will be executed during months 12-18.

Tasks 1a and 1b have been completed.
Task 1c is underway and will be completed during months 12-18
Tasks 2a-b will be completed by month 30
Tasks 3a-b will be completed by month 36

Key research accomplishments

1. Overexpression of Epidermal Growth Factor Receptor in MCF-10A human breast epithelial cells
2. Collation of a library of cell lines each representative of distinct genetic alterations of the PI3K/AKT/mTOR pathway
3. Biochemical characterization of the above cell lines is complete, phenotypical characterization is ongoing

Reportable Outcomes

The results to date will be presented at the Department of Defense Era of Hope Conference, Florida, August 2011 as a poster presentation.

Conclusions

PIK3CA mutations, EGFR overexpression, Pten loss and *AKT1* E17K mutations have different biochemical signaling profiles

Although *PIK3CA* mutations, Pten loss and *AKT1* E17K mutations were originally thought to be functionally equivalent based upon their mutual exclusivity in human cancers (5, 6), recent studies have now demonstrated differences in pathway activation among these genetic alterations as well as rare cancers that have lost Pten and concurrently harbor an activating *PIK3CA* mutation (7, 8). In order to perform comparative analyses between *PIK3CA* knock in, *PTEN* knock out, *AKT1* E17K knock in and EGFR overexpressing cells in the same MCF-10A background, we first performed western blot analyses to determine the degree of MAPK and PI3K pathway activation by comparing relative levels of phosphorylated and total Akt and Erk in the absence of exogenous EGF and in the presence of physiologic concentrations of EGF (Figure 2). Representative clones for *PIK3CA* exon 9 knock in, *PTEN* knock out and *AKT1* E17K knock in were used as these cell lines have all previously been described to be indistinct from their clonal sibs (1-3). Because EGFR overexpressing clones have not yet been characterized, all three clones were used for these studies. Previously, we have described that *AKT1* E17K knock in cells do not proliferate in the absence of EGF and concordantly, they display minimal phosphorylation of Akt and Erk (3). As seen in Fig. 2, *AKT1* E17K cells did not demonstrate any significant activation of the PI3K or MAPK pathways relative to parental MCF-10A cells, as shown by the minimal phosphorylation of Akt and Erk under EGF free and physiologic concentrations of EGF (0.2 ng/ml). It should be noted that a slight under loading of the *AKT1* E17K sample accounts for the slight decrease of all proteins in the absence of EGF (Fig.2, left panel). However, in the presence of EGF, there was a slight but reproducible increase in ERK phosphorylation in *AKT1* E17K cells relative to parental MCF-10A cells (Fig.2, right panel). The reason for this is unclear, but reaffirms the notion that signaling via EGF/EGFR can lead to unexpected and varying responses depending on the genetic alterations present within a given cell. In contrast, phosphorylated Akt was increased in the *PTEN* -/- cell line, but this was not as pronounced as in the three EGFR overexpressing clones, or the *PIK3CA* knock in cell line in conditions without exogenous EGF (Fig. 2, left panel), though was comparable to these cell lines in conditions with 0.2 ng/ml EGF (Fig. 2, right panel).

EGFR overexpressing clones, *PIK3CA* knock in cells and *PTEN* -/- cells also demonstrated activation of the MAPK pathway as displayed by the increased levels of phosphorylated Erk relative to total Erk both in the absence and presence of 0.2 ng/mL EGF (Fig. 2). Interestingly, in the absence of EGF the *PTEN* -/- cell lines exhibited a pronounced increase in Erk phosphorylation compared to the EGFR overexpressing cell lines or the *PIK3CA* knock in cell line (Fig.2, left panel). However, in the presence of EGF, Erk phosphorylation in EGFR overexpressing clones was decreased relative to *PTEN*-/- cells and *PIK3CA* knock in cells (Fig.2, right panel). The cause for these differences are unknown, but these results are consistent with our previous observations in *PTEN*-/- cell lines showing that the presence or absence of EGF as well as duration of exposure to this growth factor can influence the level of Erk phosphorylation (2). Thus, our biochemical analyses reaffirm that the presence of an *AKT1* E17K mutation alone does not confer significant oncogenic pathway signaling in human breast epithelial cells. In contrast, the presence of a *PIK3CA* oncogenic mutation, the loss of Pten, or overexpression of EGFR does indeed result in Akt and Erk phosphorylation in a manner similar to that found in breast cancer cells. However, the level and pattern of activation seen in these pathways is distinctly different between these three sets of cell lines, as evidenced by the varying levels of phosphorylation seen under conditions with and without exogenous EGF. This further underscores the previously unrecognized complexity of crosstalk that occurs between these important pathways.

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Appendices

None at this time